Deuse T, Wang D, Stubbendorff M, Itagaki R, Grabosch A, Greaves LC, Alawi M, Grunewald A, Hu XM, Hua XQ, Velden J, Reichenspurner H, Robbins RC, Jaenisch R, Weissman IL, Schrepfer S. SCNT-Derived ESCs with Mismatched Mitochondria Trigger an Immune Response in Allogeneic Hosts. *Cell Stem Cell* 2015, 16(1), 33-38.

SCNT-derived ESCs with mismatched mitochondria trigger an immune response in allogeneic hosts

Tobias Deuse[1,2,3], Dong Wang[1,2], Mandy Stubbendorff[1,2], Ryo Itagaki[1,2], Antje Grabosch[1,2], Laura C. Greaves[4], Malik Alawi[5,6], Anne Grünewald[7], Xiaoqin Hua[1,2], Joachim Velden[8], Robert C. Robbins[9], Rudolf Jaenisch[10], Irving L. Weissman*[11], Sonja Schrepfer*[1,2,9]

*I.L.W. and S.S. share last-authorship

[1]TSI-laboratory, University Heart Center Hamburg, Germany. [2]Cardiovascular Research Center Hamburg (CVRC) and DZHK (German Center for Cardiovascular Research), partner site Hamburg/Kiel/Luebeck, Germany. [3]Cardiovascular Surgery, University Heart Center Hamburg, Germany. [4]Newcastle University Centre for Brain Ageing and Vitality, Newcastle University, Newcastle upon Tyne, NE2 4HH, United Kingdom. [5]Bioinformatics Service Facility, University Medical Center Hamburg-Eppendorf, Germany. [6]Heinrich-Pette-Institute, Leibniz-Institute for Experimental Virology, Virus Genomics, Germany. [7]Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE2 4HH, United Kingdom. [8]Department of Nephropathology, Institute of Pathology, University Hospital Erlangen, Erlangen, Germany. [9]Stanford Cardiovascular Institute and Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, CA, USA. [10]Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA, USA. [11]Department of Developmental Biology, Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA.

Address for Correspondence:

Sonja Schrepfer, M.D., Ph.D.

Department of Cardiothoracic Surgery

Stanford Cardiovascular Institute

Stanford University School of Medicine

300 Pasteur Dr., CVRB MC 5407

Stanford, CA 94305, USA

Tel: (650) 736-8592; Fax: (650) 725-3846

E-mail: schrepfer@stanford.edu

Running title: Mitochondria possess alloantigenicity.

Summary

The generation of pluripotent stem cells by somatic cell nucleus transfer (SCNT) has recently been achieved in human cells and sparked new interest in this technology. The authors reporting this methodical breakthrough speculated that SCNT would allow the creation of patient-matched embryonic stem cells, even in patients with hereditary mitochondrial diseases. However, herein we show that mismatched mitochondria in nuclear transfer-derived embryonic stem cells (NT-ESC) possess alloantigenicity and are subject to immune rejection. In a murine transplantation setup, we demonstrate that allogeneic mitochondria in NT-ESCs, which are nucleus-identical to the recipient, may trigger an adaptive alloimmune response that impairs the survival of NT-ESC grafts. The immune response is adaptive, directed against mitochondrial content, and amenable for tolerance induction. Mitochondrial alloantigenicity should therefore be considered when developing therapeutic SCNT-based strategies.

Highlights

- Mismatched mitochondria in nuclear transfer-derived embryonic stem cells (NT-ESC) possess alloantigenicity and are subject to immune rejection.

- The alloimmune response against mitochondria mismatched NT-ESCs is weaker than that against major histocompatibility complex (MHC)-mismatched cell grafts.
- The immune response is adaptive, directed against mitochondrial content, and amenable for tolerance induction.

eTOC Blurb

Mismatched mitochondria in SCNT-derived ESC grafts trigger immune rejection despite matching for the entire nuclear genome with the murine recipient.

In somatic cell nucleus transfer (SCNT), the nucleus of a somatic cell is transplanted into an enucleated oocyte (Noggle et al., 2011). It has been envisioned that by this means, autologous pluripotent stem cells can be generated for specific patient-matched therapies. Embryonic stem cells by nuclear transfer (NT-ESCs) were first successfully generated from mice (Munsie et al., 2000) and primates (Byrne et al., 2007). After substantial optimization of the SCNT technique, the derivation of NT-ESCs from human fetal fibroblasts (Tachibana et al., 2013), and most recently, also from adult somatic cells (Chung et al., 2014; Yamada et al., 2014) was reported. These latter studies suggest that age-associated changes in the nucleus donor cell are not necessarily an impediment to generating human NT-ESCs (Chung et al., 2014). Furthermore, the differentiation of insulin-producing beta cells derived from NT-ESCs generated from a long-standing diabetic patient proved that specialized cell types that were lost in diseased patients could be re-established (Yamada et al., 2014). Because NT-ESCs acquire healthy mitochondria from the selected oocyte donor, inherited or acquired mtDNA diseases are most amenable for correction using the SCNT technology (Tachibana et al., 2013). The recent advancements in SCNT methodology have increased expectations for this approach to lead to human-assisted reproductive technologies (Cibelli, 2014).

A so far underappreciated obstacle for clinical application of SCNT-derived cell therapy may be the immunogenicity of mismatched mitochondria in NT-ESCs. After transplantation of NT-ESC-derived cells or tissue back into the nucleus donor, mismatched mtDNA-coded proteins may induce alloimmunity. We here show that allogeneic mitochondria in murine NT-ESCs may trigger an adaptive immune response that impairs the survival of NT-ESC grafts.

To assess mitochondria-specific alloantigenicity, transplantation studies with SCNT-derived murine NT-ESCs were conducted across defined immunological barriers. SCNT was performed as described earlier (Kirak et al., 2010) using enucleated BDF1 (B6D2F1/J) oocytes (C57BL/6J × DBA/2NCrl)F1 and isolated BALB/c (BALB/cAnNCrl; major histocompatibility complex (MHC) $H2^d$) fibroblast nuclei (Figure 1A). Both oocytes and NT-ESCs demonstrated high mitochondrial

abundance (Figure S1A). The high repetition of mtDNA copies and high amount of mitochondrial proteins contributes to their relevance as possible immunogenic antigens. Culture-expanded NT-ESCs expressed markers related to pluripotency (Figure S1B). NT-ESCs grew tumors in immunodeficient NOD SCID mice (NOD.CB17-Prkdc^{scid}/J). These tumors contained tissues of endodermal, ectodermal, and mesodermal origin (Figures S1C-D), thus meeting the criteria for teratomas. The generated NT-ESCs thus contain maternally-inherited C57BL/6J (B6) mitochondria and BALB/c nuclei of the H2^{d} haplotype. Suspensions of 10^6 NT-ESCs were intramuscularly injected into the thighs of recipient mice. BALB/c recipients are matched for the entire nuclear genome, but are mitochondria-mismatched. mtDNA sequence analysis comparing B6 mitochondria of the NT-ESCs with BALB/c mitochondria revealed 2 non-synonymous nucleotide substitutions within the *mt-Co3* and *mt-Cytb* genes, which resulted in amino acid substitutions in the associated proteins (Figure S2A). These two non-synonymous single nucleotide polymorphisms (SNPs) were therefore the only differences in the entire cellular DNA between donor cells and recipient. According to dbSNP (Sherry et al., 2001), both variants are common in mouse. The affected loci are conserved among vertebrates (Figure S2B) and for one of the two observed variants a human counterpart is described in dbSNP. BDF1 (H2$^{b/d}$) recipients share the H2^{d} alleles with the NT-ESCs, are mitochondria-matched, but minor histocompatibility antigen (MiHA)-mismatched (Figure 1A). B6 recipients possess the H2^{b} haplotype and are therefore MHC-mismatched, but mitochondria-matched to the NT-ESCs. The severely immunodeficient NOD SCID strain was used as negative control, because it cannot mount any antigen-specific cellular immune response. The CBA (CBA/J; H2^{k}) strain is both MHC and mitochondria mismatched to the NT-ESCs. mtDNA sequencing revealed identical mtDNA genomes for CBA and BALB/c, (not shown) as was previously reported in the context of mouse mtDNA phylogeny (Goios et al., 2007). Therefore, besides the MHC differences, NT-ESC transplantations into BALB/c and CBA cross the same mitochondrial mismatch.

Previous studies on the immunogenicity of murine mitochondrial peptides were mostly restricted to cytotoxic T lymphocytes (CTLs) and MHC class Ia and Ib presentation (Dabhi and Lindahl, 1995). We herein used assays that quantify activation of T helper cells (Th), because this entity plays a central role in activating CTLs and B cells, thus orchestrating graft rejection. Five-day ELISPOT assays for Th1-specific γ-interferon (IFNγ) showed a substantial immune activation in the MHC-mismatched B6 recipients, which was significantly stronger than those in mitochondria-mismatched BALB/c or MiHA-mismatched BDF1 (Figure 1B). The MHC- and mitochondria-mismatched CBA strain demonstrated the highest spot frequencies of all strains, which were approximately additive of the frequencies of the isolated MHC-mismatched B6 and the isolated mitochondria-mismatched BALB/c. The immunodeficient NOD SCID recipients showed immunological Th1 anergy. The Th2 response, although generally generating lower IL-4 spot frequencies, showed a similar relation between the recipient strains (Figure 1C). There was also a surge in NT-ESC-specific IgM antibodies 5 days after immunization in B6 recipients, which was significantly stronger than those in BALB/c or BDF1 (Figure 1D). There were no NT-ESC-specific IgM antibodies in NOD SCID.

In an additional experiment, we could exclude that there were general differences among BALB/c, BDF1, and B6 in the degree of immune responsiveness (Figure S2C). Splenocytes of these three strains were unspecifically stimulated and ELISPOT assays demonstrated similar Th1 and Th2 activation. This result permits us to attribute the above ELISPOT results to the mismatched proteins.

To exclude methodological bias by the SCNT technique itself, isogeneic BALB/c ESCs (isoESCs) were generated using SCNT with BALB/c donors for both oocytes and nuclei (Figure 1E). Transplantation experiments were performed with fully nuclear DNA- and mtDNA-matched, isogeneic BALB/c recipients, MiHA- and mitochondria-mismatched BDF1, and MHC- and mitochondria-mismatched B6. There was negligible Th1 and Th2 activation in isogeneic BALB/c recipients (Figure 1F and G). Between mitochondria-mismatched recipients, an additional MHC

mismatch (B6) generated a significantly stronger Th1 activation than an additional MiHA mismatch (BDF1, Figure 1F). Together, these results demonstrate that an isolated mitochondria mismatch is sufficient to initiate marked Th activation and NT-ESC-directed antibody production. These immune responses are not caused by the SCNT technology itself. Second, an MHC mismatch causes a significantly stronger immune activation than a MiHA mismatch, both in the setting of additional mitochondrial match or mismatch. And third, the Th1 activation is additive for an MHC- and a mitochondria-mismatch.

Based on the results by Ishikawa (Ishikawa et al., 2010), which assumed that the innate immunity was responsible for rejection of mitochondria-mismatched cell grafts, the T cell-deficient nude mouse model was used to study the NK cell response. BALB/c nude (BALB/c nu/nu, CAnN.Cg-Foxn1<nu>/Crl; $H2^d$) were used as recipients for either mitochondria-mismatched NT-ESCs or fully isogeneic isoESCs ($H2^d$, Figure 1H). Our results show that there was no increase in NK cell-driven IFNγ ELISPOT frequency in response to NT-ESCs compared to isoESCs (Figure 1I). Also, the survival of cell grafts was similar for NT-ESCs and isoESCs and all animals formed teratomas, thus excluding relevant innate rejection processes (Figure 1J-K). Importantly, the transplantation experiment by Ishikawa was specifically designed to be mismatched for the maternally transmitted mitochondrial antigen (Mta) previously described by Fischer Lindahl (Fischer Lindahl et al., 1980). Our transplant setting avoids this incompatibility and, as a consequence, we did not observe relevant innate immunity-driven rejection.

Survival of NT-ESC grafts and teratoma development were assessed in the transplantation settings of Figure 1A. As expected, all NT-ESC transplants survived and formed teratomas in NOD SCID recipients (Figure 2A), whereas all grafts were rejected in fully mismatched CBAs (Figure 2B). The rather mild Th1 activation in MiHA-mismatched BDF1 still allowed for 90% teratoma development of rapidly-proliferating NT-ESC grafts (Figure 2C), whereas there was only 20% teratoma growth in MHC-mismatched B6 (Figure 2D). In mitochondria-mismatched BALB/c, teratomas developed in 60% of the recipients, but the tumor growth was markedly

slowed (Figure 2E). Half of the tumors did not develop until day 50 to 90. Interestingly, there was a strong correlation between graft survival and Th1 activation in individual BALB/c animals (Figure 2F). Only BALB/c recipients with very low spot frequencies developed teratomas, whereas animals with at least moderate Th1 activation rejected their grafts. This further supports the notion of a T cell-mediated rejection process of mitochondria-mismatched grafts.

To confirm a mitochondrial location of the immunogenic peptides, mitochondria of NT-ESCs were separated from the remainder of the cell (cytosol) and splenocytes of NT-ESC-immunized BALB/c were re-stimulated in vitro with either mismatched mitochondria or isogeneic cytosol (Figure 2G). Only the mismatched mitochondria caused an IFNγ response, whereas the spot frequencies with the isogeneic cytosol fractions were low, similar to those of un-stimulated responder splenocytes (Figure 2H). Of note, the ELISPOT frequencies of the separated cell compartments were markedly lower than those involving whole cells. Although their precise presentation mechanism remains elusive, the detection of mitochondrial peptides in the context of MHC (Dabhi and Lindahl, 1995; Duvvuri et al., 2014) suggests they enter conventional MHC antigen presentation pathways. The dependence on indirect antigen presentation in this assay may explain this observation. In accordance with the ELISPOT results, in a delayed-type hypersensitivity test, only mismatched mitochondria generated paw swelling, whereas isogeneic cytosol did not (Figure 2I). The location of the antigens for BALB/c immune activation against NT-ESCs was thus restricted to the mitochondrial fraction.

We next tested whether acquired immunological tolerance against allogeneic mitochondria can be induced. In accordance with the Medawar experiments (Billingham et al., 1953), neonatal BALB/c mice were immunized with mitomycin-inhibited NT-ESCs (Figure 2J). In adulthood, these animals were re-injected with NT-ESCs ($BALB/c_{re\text{-}inject}$). In contrast to adult BALB/c recipients that received their first NT-ESC grafts, $BALB/c_{re\text{-}inject}$ showed no IFNγ response in ELISPOT assays and all animals developed teratomas (Figure 2K-L). This experiment not only shows that tolerance against allogeneic mitochondria can reliably be induced, it is also further

proof for the relevance of the adaptive immune system in rejecting mitochondria-mismatched grafts.

There have been few publications on the transplantation of mitochondria-mismatched cells or tissues with conflicting results. Although prolonged hematopoietic chimerism (Lanza et al., 2005) and acceptance of cardiac and renal cell transplants (Lanza et al., 2002) in cows receiving SCNT-derived mitochondria-mismatched cells was reported, this may not guarantee universal success. Pigs rejected kidney grafts from SCNT-derived mitochondria-mismatched clones (Kwak et al., 2013). In mice and rats, it was shown that a single non-synonymous nucleotide substitution in the mitochondrial ND1 gene or the ATPase 6 gene, respectively, generated an aberrant peptide and resulted in the loss of histocompatibility (Bhuyan et al., 1997; Davies et al., 1991; Loveland et al., 1990). Neopeptides from human mtDNA harboring large-scale frameshift deletions were recently reported to bind to MHC molecules and can be recognized by T cells from other individuals (Duvvuri et al., 2014). Interestingly, their results further suggest that some neopeptides could bind promiscuously to both MHC class I and class II alleles, activate CD4 as well as CD8 T cells, and be recognized by CD8 memory T cells. Aberrant mitochondrial peptides may even provoke MHC overexpression and thus amplify their immunogenicity. In both mice and humans, increased MHC class I expression in cells harboring mtDNA deletions was shown (Gu et al., 2003). Mitochondrial peptides can thus serve as immunogens (Morse et al., 1996) and the recipient immune response may depend on the alloantigenicity of the aberrant mitochondrial protein. Although thought to be beneficial for immune surveillance and clearance of cells with mitochondrial mutations (Duvvuri et al., 2014; Gu et al., 2003), this immune feature is potentially hazardous in the context of SCNT-derived cell transplantation.

Artificial reprograming using viral or non-viral plasmid-based approaches seems to alter the expression of a multitude of genes in induced pluripotent stem cells (iPSCs) that are differentially expressed in ESCs (de Almeida et al., 2014; Zhao et al., 2011). Specific factors

being suppressed (de Almeida et al., 2014) or overexpressed (Zhao et al., 2011) in the pluripotent state have been suggested to contribute to the antigenicity of iPSCs. With differentiation, the antigenicity was reported to vanish as transcriptome profile and surface antigen expression converged with those of corresponding somatic cells (de Almeida et al., 2014). Terminally differentiated cells derived from iPSCs are now believed to possess no or negligible immunogenicity (Araki et al., 2013). The described immunogenicity is therefore differentiation-dependent and not due to antigenic somatic coding mutations, which have recently been reported to occur during reprogramming (Gore et al., 2011). When compared to autologous iPSCs that are fully matched for both nuclear and mitochondrial DNA, NT-ESCs with mtDNA differences to the nucleus donor possess additional allogeneic peptides that may potentially be antigenic. These antigenic proteins in mitochondria-mismatched NT-ESCs are maintained with differentiation and the mtDNA copies, if anything, are increased in energy-consuming differentiated cells like cardiomyocytes. However, it remains to be determined if the results presented herein are transferrable to differentiated cells and tissues from NT-ESCs.

The level of heterogeneity in human mitochondria is considerably higher than that in laboratory mice (Goios et al., 2007); dbSNP lists 2066 active human mitochondrial RefSNPs. At least 64 thereof are common (global minor allele frequency 1% or higher) and non-synonymously coding. *MT-CYTB* is affected by 26 such variants and *MT-CO3* by two, including the human counterpart of the murine variant we observed. A recent study hints at the level of heterogeneity in human mitochondrial genomes (Ridge et al., 2014). Within a dataset of 1007 full mitochondrial genomes, 899 single nucleotide variants, 26 insertions, and 20 deletions were identified. Compared to the human reference sequence, each individual carried on average 25.3 variants. Single individuals marked the extremes with 2 and 52 variants, respectively.

In the field of xenotransplantation, the immunity against the αGal-antigen was long considered to be the one obstacle that needed to be overcome to clear the path for subsequent clinical application. However, when αGal was knocked out, xeno-immune responses were still evident

and new immune targets surfaced that previously were overshadowed by the strong αGal response (Miyata and Platt, 2003). Similarly, when nuclear DNA is matched in SCNT-derived cells, antigenic competition (Johnson et al., 1981) may allow mitochondrial antigens to emerge as immunogens. The immunogenicity of mitochondria should thus not be neglected when advancing the SCNT technology.

References

Araki, R., Uda, M., Hoki, Y., Sunayama, M., Nakamura, M., Ando, S., Sugiura, M., Ideno, H., Shimada, A., Nifuji, A*., et al.* (2013). Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature *494*, 100-104.

Bhuyan, P.K., Young, L.L., Lindahl, K.F., and Butcher, G.W. (1997). Identification of the rat maternally transmitted minor histocompatibility antigen. J Immunol *158*, 3753-3760.

Billingham, R.E., Brent, L., and Medawar, P.B. (1953). Actively acquired tolerance of foreign cells. Nature *172*, 603-606.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., Nelson, M., Sanger, W.G., Gokhale, S., Wolf, D.P., and Mitalipov, S.M. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature *450*, 497-502.

Chung, Y.G., Eum, J.H., Lee, J.E., Shim, S.H., Sepilian, V., Hong, S.W., Lee, Y., Treff, N.R., Choi, Y.H., Kimbrel, E.A*., et al.* (2014). Human somatic cell nuclear transfer using adult cells. Cell Stem Cell *14*, 777-780.

Cibelli, J.B. (2014). Human somatic cell nuclear transfer is alive and well. Cell Stem Cell *14*, 699-701.

Dabhi, V.M., and Lindahl, K.F. (1995). MtDNA-encoded histocompatibility antigens. Methods Enzymol *260*, 466-485.

Davies, J.D., Wilson, D.H., Hermel, E., Lindahl, K.F., Butcher, G.W., and Wilson, D.B. (1991). Generation of T cells with lytic specificity for atypical antigens. I. A mitochondrial antigen in the rat. J Exp Med *173*, 823-832.

de Almeida, P.E., Meyer, E.H., Kooreman, N.G., Diecke, S., Dey, D., Sanchez-Freire, V., Hu, S., Ebert, A., Odegaard, J., Mordwinkin, N.M*., et al.* (2014). Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat Commun *5*, 3903.

Duvvuri, B., Duvvuri, V.R., Wang, C., Chen, L., Wagar, L.E., Jamnik, V., Wu, J., Yeung, R.S., Grigull, J., Watts, T.H*., et al.* (2014). The human immune system recognizes neopeptides derived from mitochondrial DNA deletions. J Immunol *192*, 4581-4591.

Fischer Lindahl, K., Bocchieri, M., and Riblet, R. (1980). Maternally transmitted target antigen for unrestricted killing by NZB T lymphocytes. J Exp Med *152*, 1583-1595.

Goios, A., Pereira, L., Bogue, M., Macaulay, V., and Amorim, A. (2007). mtDNA phylogeny and evolution of laboratory mouse strains. Genome Res *17*, 293-298.

Gore, A., Li, Z., Fung, H.L., Young, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E*., et al.* (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature *471*, 63-67.

Gu, Y., Wang, C., Roifman, C.M., and Cohen, A. (2003). Role of MHC class I in immune surveillance of mitochondrial DNA integrity. J Immunol *170*, 3603-3607.

Ishikawa, K., Toyama-Sorimachi, N., Nakada, K., Morimoto, M., Imanishi, H., Yoshizaki, M., Sasawatari, S., Niikura, M., Takenaga, K., Yonekawa, H*., et al.* (2010). The innate immune system in host mice targets cells with allogenic mitochondrial DNA. J Exp Med *207*, 2297-2305.

Johnson, L.L., Bailey, D.W., and Mobraaten, L.E. (1981). Antigenic competition between minor (non-H-2) histocompatibility antigens. Immunogenetics *13*, 451-455.

Kirak, O., Frickel, E.M., Grotenbreg, G.M., Suh, H., Jaenisch, R., and Ploegh, H.L. (2010). Transnuclear mice with predefined T cell receptor specificities against Toxoplasma gondii obtained via SCNT. Science *328*, 243-248.

Kwak, H.H., Park, K.M., Teotia, P.K., Lee, G.S., Lee, E.S., Hong, S.H., Yang, S.R., Park, S.M., Ahn, C., Park, C.K.*, et al.* (2013). Acute rejection after swine leukocyte antigen-matched kidney allo-transplantation in cloned miniature pigs with different mitochondrial DNA-encoded minor histocompatibility antigen. Transplant Proc *45*, 1754-1760.

Lanza, R., Shieh, J.H., Wettstein, P.J., Sweeney, R.W., Wu, K., Weisz, A., Borson, N., Henderson, B., West, M.D., and Moore, M.A. (2005). Long-term bovine hematopoietic engraftment with clone-derived stem cells. Cloning Stem Cells *7*, 95-106.

Lanza, R.P., Chung, H.Y., Yoo, J.J., Wettstein, P.J., Blackwell, C., Borson, N., Hofmeister, E., Schuch, G., Soker, S., Moraes, C.T.*, et al.* (2002). Generation of histocompatible tissues using nuclear transplantation. Nat Biotechnol *20*, 689-696.

Loveland, B., Wang, C.R., Yonekawa, H., Hermel, E., and Lindahl, K.F. (1990). Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. Cell *60*, 971-980.

Miyata, Y., and Platt, J.L. (2003). Xeno--still stuck without alphaGal. Nat Biotechnol *21*, 359-360.

Morse, M.C., Bleau, G., Dabhi, V.M., Hetu, F., Drobetsky, E.A., Lindahl, K.F., and Perreault, C. (1996). The COI mitochondrial gene encodes a minor histocompatibility antigen presented by H2-M3. J Immunol *156*, 3301-3307.

Munsie, M.J., Michalska, A.E., O'Brien, C.M., Trounson, A.O., Pera, M.F., and Mountford, P.S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr Biol *10*, 989-992.

Noggle, S., Fung, H.L., Gore, A., Martinez, H., Satriani, K.C., Prosser, R., Oum, K., Paull, D., Druckenmiller, S., Freeby, M.*, et al.* (2011). Human oocytes reprogram somatic cells to a pluripotent state. Nature *478*, 70-75.

Ridge, P.G., Maxwell, T.J., Foutz, S.J., Bailey, M.H., Corcoran, C.D., Tschanz, J.T., Norton, M.C., Munger, R.G., O'Brien, E., Kerber, R.A.*, et al.* (2014). Mitochondrial genomic variation associated with higher mitochondrial copy number: the Cache County Study on Memory Health and Aging. BMC Bioinformatics *15 Suppl 7*, S6.

Sherry, S.T., Ward, M.H., Kholodov, M., Baker, J., Phan, L., Smigielski, E.M., and Sirotkin, K. (2001). dbSNP: the NCBI database of genetic variation. Nucleic Acids Res *29*, 308-311.

Tachibana, M., Amato, P., Sparman, M., Gutierrez, N.M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H.S., Sritanaudomchai, H.*, et al.* (2013). Human embryonic stem cells derived by somatic cell nuclear transfer. Cell *153*, 1228-1238.

Yamada, M., Johannesson, B., Sagi, I., Burnett, L.C., Kort, D.H., Prosser, R.W., Paull, D., Nestor, M.W., Freeby, M., Greenberg, E.*, et al.* (2014). Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. Nature *510*, 533-536.

Zhao, T., Zhang, Z.N., Rong, Z., and Xu, Y. (2011). Immunogenicity of induced pluripotent stem cells. Nature *474*, 212-215.

Author Contributions

T.D., R.C.R., R.J., I.L.W., and S.S. designed the study. T.D., D.W., M.A., and S.S. analyzed the data and performed the statistics. D.W. performed the live cell mitochondrial staining, characterized the cells, and provided all confocal data. M.S., R.I., and Antje G. performed the ELISPOT assays and generated the cell survival data. M.S. contributed to the FACS and DTH data. L.C.G. and Anne G. performed the mtDNA sequencing. M.A. performed bioinformatics analyses of mtDNA sequences. X.H. and J.V. generated the teratoma data. R.J. generated the SCNT cells. S.S. performed or supervised cell transplantation and all immunologic assays. T.D., D.W., M.A., and S.S. wrote the manuscript and all authors edited the manuscript.

Acknowledgements

We thank Kirsten Fischer Lindahl for her critical comments and editing of the manuscript. We further thank Christiane Pahrmann for performing all cell cultures and for her overall technical assistance. Special thanks to the UKE Imaging Facility (UMIF, Bernd Zobiak) and the UKE Animal Facility.

This study was supported by the Else-Kröner-Fresenius-Stiftung (2012_EKES.04; T.D.), a grant from the Fondation Leducq (CDA 2013-2015; S.S.), and the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG: SCHR992/3-1 and SCHR992/4-1).

The authors have no conflicts of interest to disclose.

Figure Legends

Figure 1: *NT-ESCs possess mitochondria-related antigenicity.*

(A) NT-ESCs were generated using enucleated BDF1 oocytes and isolated BALB/c fibroblast nuclei. NT-ESCs were transplanted into mitochondria-MM BALB/c, MiHA-MM BDF1, MHC-MM B6, immunodeficient NOD SCID, or combined MHC- and mitochondria-MM CBA. See also Figure S1.

(B) Recipient-specific Th1 ELISPOT results against NT-ESCs showed significantly lower IFNγ spot frequencies for BALB/c (n=18) and BDF1 recipients (n=17) compared to B6 (n=24). Overall, the CBA IFNγ response (n=15) was the strongest and the NOD SCID response (n=6) negligible.

(C) IL-4 (Th2) ELISPOT responses revealed similar relations among groups (BALB/c n=12, BDF1 n=17, B6 n=24, NOD SCID n=8, CBA n=10) as described in (B) for Th1 response.

(D) The NT-ESC-specific IgM antibody production after 5 days was significantly stronger in B6 (n=22) than in BALB/c (n=31) or BDF1 (n=15). No IgM antibodies against NT-ESCs were detected in NOD SCID (n=6).

(E) Isogeneic SCNT-derived BALB/c ESCs (isoESCs) were generated from BALB/c oocytes and nuclei. isoESCs were transplanted either into isogeneic BALB/c or recipients with combined mitochondria- and MiHA-MM (BDF1) or mitochondria- and MHC-MM (B6).

(F) The IFNγ ELISPOT responses against isoESC grafts were negligible in BALB/c (n=5) and significantly stronger in BDF1 (n=22) or B6 (n=6). Th1 activation in MHC-MM B6 was significantly stronger than in MiHA-MM BDF1.

(G) The isoESC-specific IL-4 ELISPOT responses were significantly stronger in BDF1 (n=23) and B6 (n=10) than in BALB/c (n=5).

(H) BALB/c nude mice received NT-ESC (mitochondria-MM) or isoESC grafts. Because of the T cell deficiency, the nude model assesses NK cell responses.

(I) The IFNγ NK cell response against NT-ESCs (n=9) was similarly low as that against isoESCs (n=8).

(J-K) Teratoma growth diagrams for NT-ESCs (J, n=5) and isoESCs (K, n=5) in BALB/c nude mice are shown. The overall teratoma development is depicted in separate adjacent graphs.

On each box in ELISPOT blots, the central mark is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points. MM=mismatch, * $p<0.05$.

Figure 2: *The rejection of mitochondria-MM NT-ESCs is adaptive, directed against mitochondrial content, and amenable for tolerance induction.*

(A-E) Teratoma growth diagrams for NT-ESC grafts in immunodeficient NOD SCID (A, n=5), combined MHC- and mitochondria-MM CBA (B, n=5), MiHA-MM BDF1 (C, n=10), MHC-MM B6 (D, n=10), and mitochondria-MM BALB/c recipients (E, n=10). The overall teratoma development is depicted in separate adjacent graphs. See also Figure S2.

(F) Within the BALB/c recipient group, the IFNγ ELISPOT frequencies of animals that did not (no teratoma, n=20) or did show teratoma growth (teratoma, n=8) were compared.

(G) BALB/c received mitochondria-MM NT-ESC grafts. Subsequent ELISPOT assays did not use NT-ESCs, but isolated mitochondria or the remainder of the cells (referred to as cytosol) for stimulation.

(H) IFNγ ELISPOT analyses show some BALB/c response against isolated NT-ESC mitochondria (NT-ESCmito, n=8), whereas no reactivity against the mitochondria-depleted NT-ESC remnants (NT-ESCcytosol, n=4) was detectable.

(I) There also was a paw swelling response in BALB/c recipients challenged with cutaneous injections of isolated NT-ESC mitochondria (n=9), but not in BALB/c challenged with the mitochondria-depleted NT-ESC remnants (n=5).

(J) One group of BALB/c mice received their first NT-ESC grafts at neonatal stage and was re-injected during adulthood (BALB/$c_{re\text{-}inject}$). The other group received NT-ESC grafts only after entering adulthood (BALB/c).

(K) In ELISPOT assays, BALB/$c_{re\text{-}inject}$ (n=14) showed a significantly lower IFNγ response than BALB/c (n=7).

(L) The overall teratoma development of NT-ESC grafts in BALB/$c_{re\text{-}inject}$ (n=27) and BALB/c animals (n=7) is presented.

On each box in ELISPOT blots, the central mark is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points. In each scatter blot, the central mark is the median. MM=mismatch, † $p<0.01$.

Figure 1

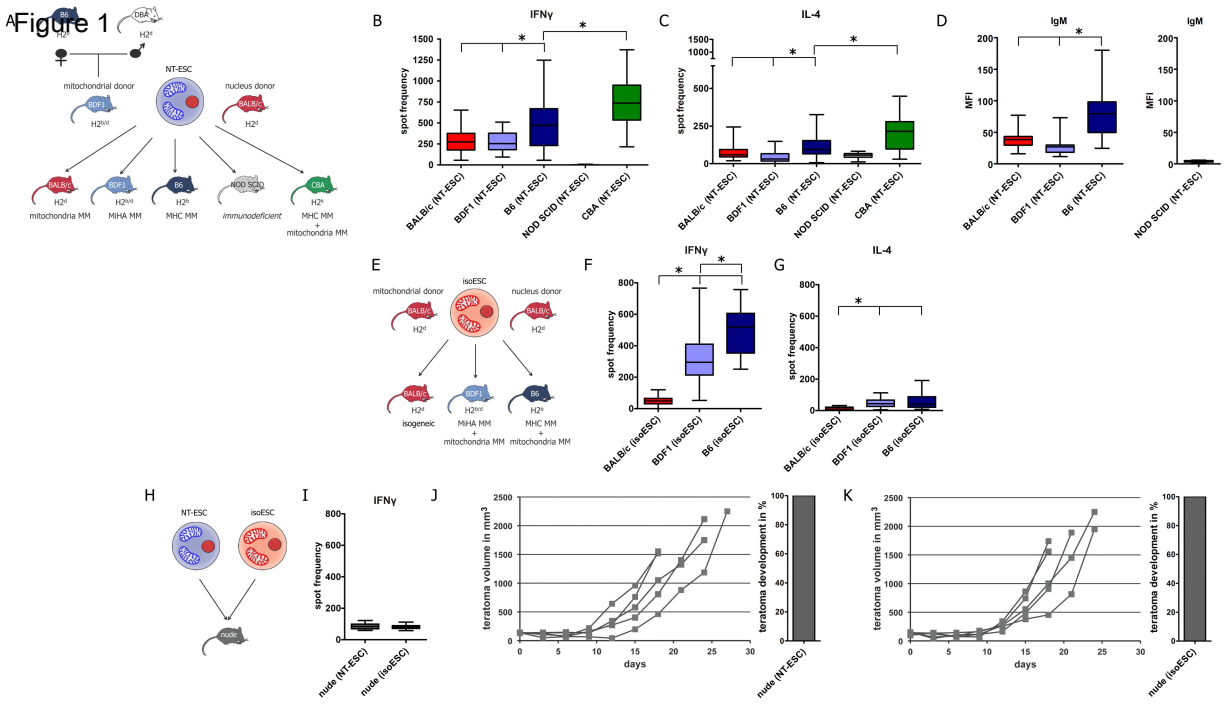
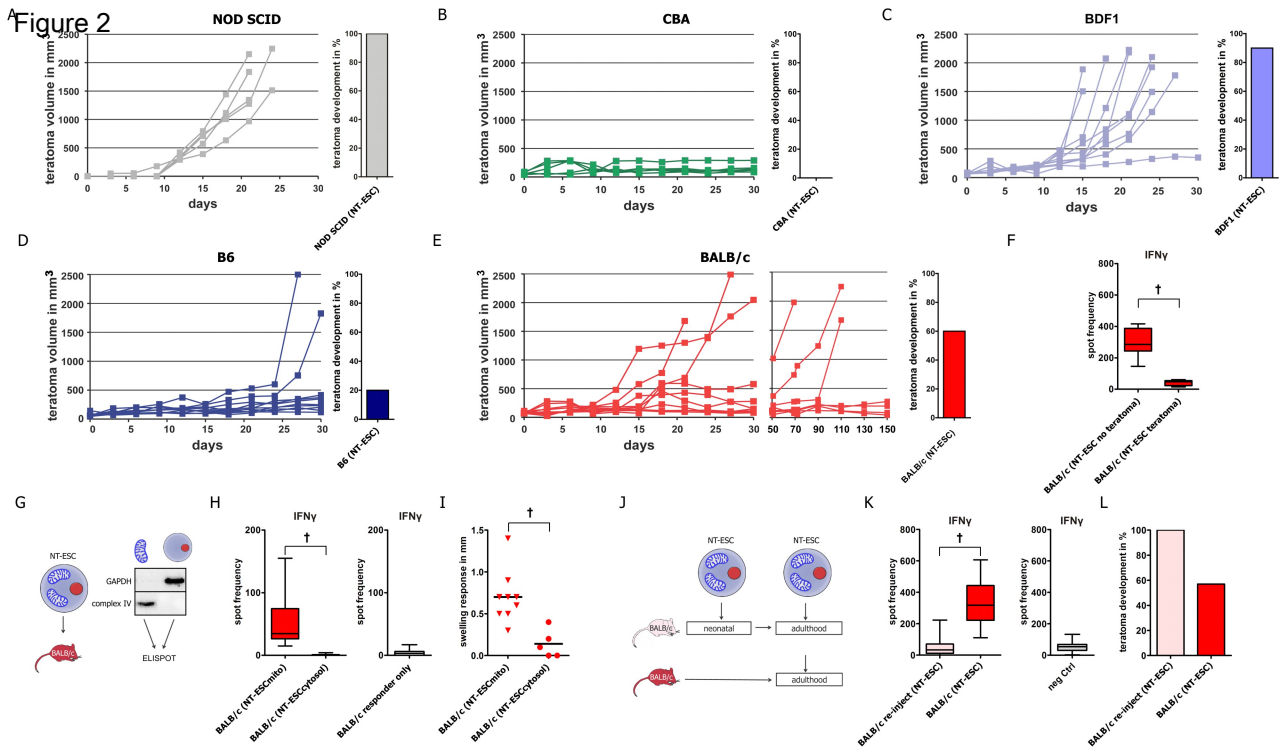

A
B6
H2b
DBA
H2d
mitochondrial donor
BDF1
H2b/d
NT-ESC
nucleus donor
BALB/c
H2d
BALB/c
H2d
BDF1
H2b/d
B6
H2b
NOD SCID
H2g7
CBA
H2k
mitochondria MM
MiHA MM
MHC MM
immunodeficient
MHC MM
+
mitochondria MM
B
IFNγ
spot frequency
1500
1250
1000
750
500
250
0
BALB/c (NT-ESC)
BDF1 (NT-ESC)
B6 (NT-ESC)
NOD SCID (NT-ESC)
CBA (NT-ESC)
C
IL-4
spot frequency
1500
1000
500
250
0
BALB/c (NT-ESC)
BDF1 (NT-ESC)
B6 (NT-ESC)
NOD SCID (NT-ESC)
CBA (NT-ESC)
D
IgM
MFI
200
150
100
50
0
BALB/c (NT-ESC)
BDF1 (NT-ESC)
B6 (NT-ESC)
IgM
MFI
200
150
100
50
0
NOD SCID (NT-ESC)
E
isoESC
mitochondrial donor
BALB/c
H2d
nucleus donor
BALB/c
H2d
BALB/c
H2d
isogeneic
BDF1
H2b/d
MiHA MM
+
mitochondria MM
B6
H2b
MHC MM
+
mitochondria MM
F
IFNγ
spot frequency
800
600
400
200
0
BALB/c (isoESC)
BDF1 (isoESC)
B6 (isoESC)
G
IL-4
spot frequency
800
600
400
200
0
BALB/c (isoESC)
BDF1 (isoESC)
B6 (isoESC)
H
NT-ESC
isoESC
nude
I
IFNγ
spot frequency
800
600
400
200
0
nude (NT-ESC)
nude (isoESC)
J
teratoma volume in mm3
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
days
teratoma development in %
100
80
60
40
20
0
nude (NT-ESC)
K
teratoma volume in mm3
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
days
teratoma development in %
100
80
60
40
20
0
nude (isoESC)

Figure 2


A
NOD SCID
teratoma volume in mm3
days
teratoma development in %
NOD SCID (NT-ESC)
B
CBA
CBA (NT-ESC)
C
BDF1
BDF1 (NT-ESC)
D
B6
B6 (NT-ESC)
E
BALB/c
BALB/c (NT-ESC)
F
IFNγ
spot frequency
BALB/c (NT-ESC no teratoma)
BALB/c (NT-ESC teratoma)
G
NT-ESC
GAPDH
complex IV
ELISPOT
BALB/c
H
IFNγ
spot frequency
BALB/c (NT-ESCmito)
BALB/c (NT-ESCcytosol)
BALB/c responder only
I
swelling response in mm
BALB/c (NT-ESCmito)
BALB/c (NT-ESCcytosol)
J
NT-ESC
neonatal
adulthood
BALB/c
K
IFNγ
spot frequency
BALB/c re-inject (NT-ESC)
BALB/c (NT-ESC)
neg Ctrl
L
teratoma development in %
BALB/c re-inject (NT-ESC)
BALB/c (NT-ESC)

Supplemental Information

Inventory of Supplemental Information:

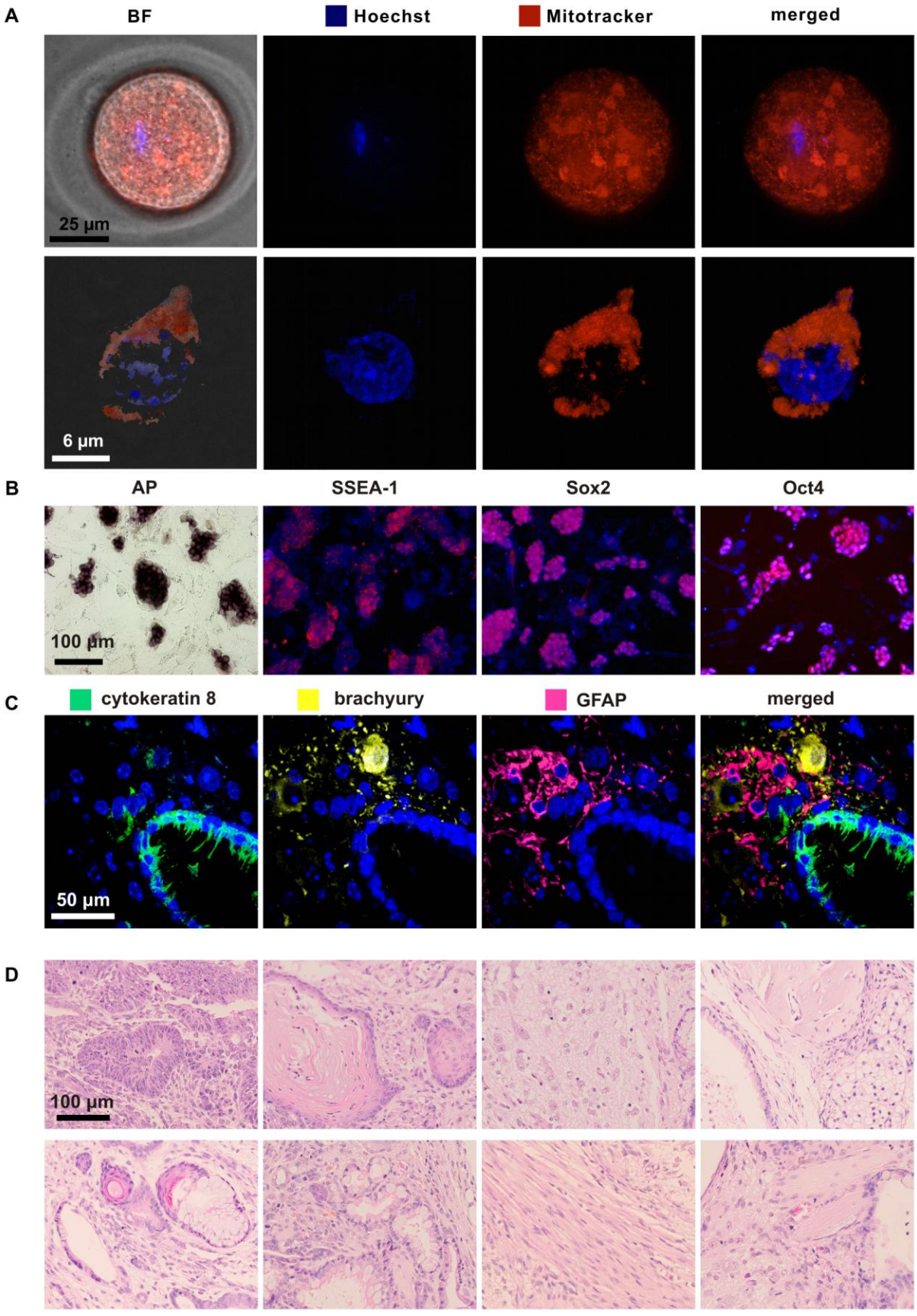

A
BF
Hoechst
Mitotracker
merged
25 μm
6 μm
B
AP
SSEA-1
Sox2
Oct4
100 μm
C
cytokeratin 8
brachyury
GFAP
merged
50 μm
D
100 μm

Figure S1: *Characterization of SCNT-derived NT-ESCs and proof of pluripotency.*

(A) A murine oocyte (upper row) and an NT-ESC (lower row) were stained for nuclear (Hoechst) and mitochondrial content (Mitotracker). Confocal immunofluorescence shows the defined nucleus and the great abundance of mitochondria throughout the cells.

(B) SCNT-derived NT-ESCs demonstrated pluripotency markers as they were positive for alkaline phosphatase (AP), and expressed SSEA-1, Sox2, and Oct4.

(C) NT-ESCs were transplanted into immunodeficient NOD SCID mice and grew tumors within 3-4 weeks. Recovered tumors stained positive for tissues of all three germ layers and were thus identified as teratomas. Endodermal (cytokeratin 8), mesodermal (brachyury), and ectodermal (GFAP) lineages were demonstrated by confocal immunofluorescence microscopy.

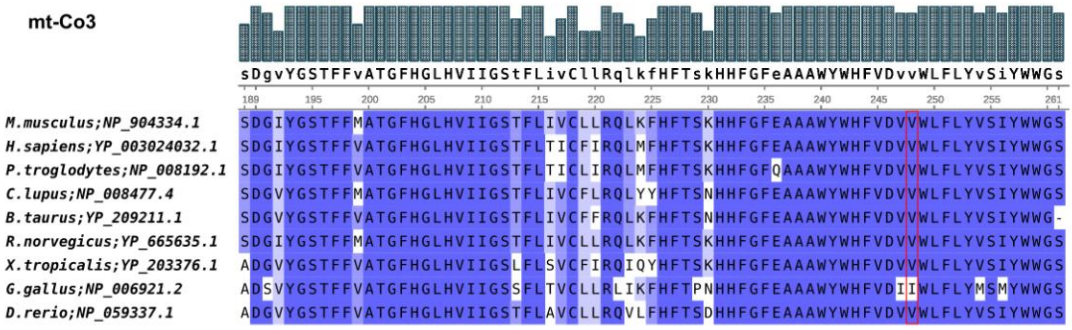
(D) Hematoxylin-eosin staining of NT-ESC-derived teratomas revealed their composition of various types of tissues. Upper row (left to right): primitive neural tube differentiation (ectoderm), squamous epithelium (ectoderm), brain tissue (ectoderm), bronchial and clear cell bronchogenic epithelium and bone (endoderm and mesoderm). Lower row (left to right): hair follicles with sebaceous glands (ectoderm), intestine (endoderm), smooth muscle (mesoderm), striated muscle (mesoderm).

A

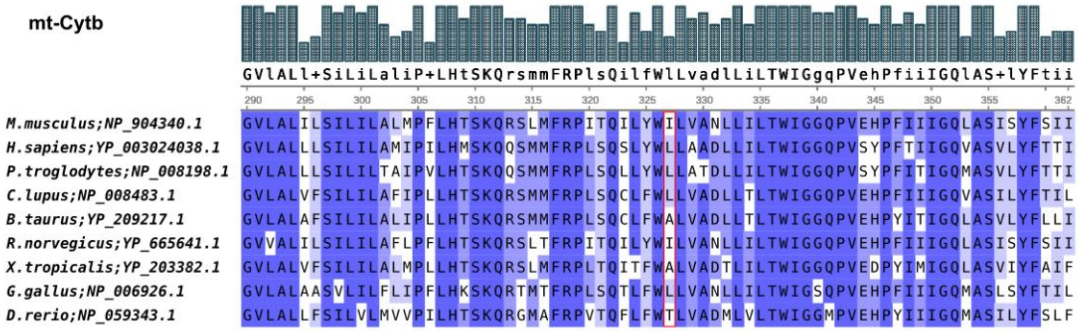
Position	Gene	Protein	Nucleotide	AA	Nucleotide	AA	RefSNP ID	RefSNP Allelic Frequency	RefSNP ID (human counterpart)
m.9348	*mt-Co3*	cytochrome c oxidase III	G	V	A	I	rs8281486	G: 61.111% (22 / 36) A: 38.889% (14 / 36)	rs370688668
m.15123	*mt-Cytb*	cytochrome b	G	V	A	I	rs33258689	G: 8.333% (2 / 24) A: 91.667% (22 / 24)	-

B

mt-Co3



mt-Cytb



C

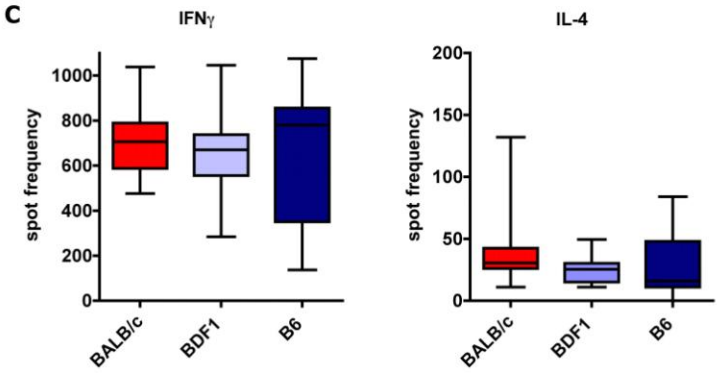


Figure S2: *Mitochondrial DNA (mtDNA) sequencing, level of conservation among vertebrates, and unspecific immune responsivenesses of different mouse strains.*

(A) The comparison of mtDNA sequences between isolated B6 mitochondria and BALB/c mitochondria revealed two non-synonymous nucleotide substitutions.

(B) Multiple sequence alignments of mt-Co3 (upper part) and mt-Cytb (lower part). The level of conservation for each position is indicated by bars and background colors. The positions affected by variants observed in this study are highlighted. Alignments were calculated using Muscle (v3.8.31) (Edgar, 2004) with default parameters. For visualization, UGENE (v1.11.5) (Okonechnikov et al., 2012) was employed.

(C) BALB/c (n=10), BDF1 (n=10 for IFNγ and n=5 for IL-4), and B6 (n=10) splenocytes were stimulated with PMA and ionomycin for 24h. The IFNγ and IL-4 ELISPOT frequencies were similar among the mouse strains.

Experimental Procedures

Generation and culture of NT-ESCs and isoESCs

Somatic cell nuclear transfer (SCNT)

Oocytes from superovulated BDF1 or BALB/c mice were metaphase-II arrested and enucleated. Nuclei from BALB/c were transferred into the enucleated oocytes to create NT-ESCs and isoESCs, respectively.

Cell culture

NT-ESCs and isoESCs were cultured on mitomycin-inhibited CF1 feeders using standard ES cell media (Gibco, Darmstadt, Germany) containing LIF (Millipore, Billerica, MA). Before in vivo cell injection and in vitro immunology experiments, NT-ESCs and isoESCs were cultured on gelatin (Millipore) without feeders using standard media containing LIF. Cell cultures were regularly screened for mycoplasma infections (Lonza, Cologne, Germany).

Mice

BALB/c (BALB/cAnNCrl, $H2^{d}$), BDF1 (B6D2F1/J, $H2^{b/d}$), C57BL/6 (C57BL/6J, B6, $H2^{b}$), NOD SCID (NOD.CB17-$Prkdc^{scid}$/J), CBA (CBA/J, $H2^{k}$), and BALB/c nude (BALB/c NU/NU, CAnN.Cg-Foxn1<nu>/Crl, $H2^{d}$) (all 6-10 weeks of age) were used as recipients for NT-ESC or isoESC grafts. Mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and received humane care in compliance with the Guide for the Principles of Laboratory Animals. Mouse studies were approved by the Hamburg “Amt für Gesundheit und Verbraucherschutz”. The numbers of mice per experimental group are presented with each figure.

Immunostaining in vitro

The mouse ESC/iPSC Characterization Kit (Applied StemCell, Sunnyvale, CA) was used to demonstrate the expression of the pluripotency markers alkaline phosphatase (AP), SSEA-1, Sox2, and Oct4. Staining was performed according to the manufacturer’s protocol.

In vivo teratoma assay

Teratoma formation was observed within 4 weeks after injecting 10^{7} NT-ESCs intramuscularly into NOD SCID mice and teratomas were fixed with 4% paraformaldehyde and paraffin-embedded for confocal immunofluorescence and histopatholgy.

To demonstrate pluripotency using immunostainings, 5µm sections underwent heat-induced antigen retrieval with Dako antigen retrieval solution (Dako, Glostrup, Denmark) in a steamer followed by blocking with Image-iT® FX signal enhancer (Invitrogen, Carlsbad, CA). Sections

were incubated with a primary antibody against brachyury (ab20680, Abcam, Cambridge, England). For detection, a goat anti-rabbit antibody labeled with Alexa Fluor 555 (Invitrogen) was used. Subsequently, sections were incubated with FITC- and Alexa Fluor 647-conjugated antibodies against cytokeratin 8 (SB37b, Abcam) and GFAP (GA5, Cell signaling, Cambridge, UK), respectively. After nuclei staining with DAPI, images were obtained and analyzed using the Nikon Eclipse TiE microscope (Nikon, Tokyo, Japan) equipped with Perkin Elmer UltraVIEW VoX confocal imaging system (Perkin Elmer, Waltham, MA). Hematoxylin and eosin (H&E) stained slides were interpreted by an expert pathologist (J.V.) blinded to the study.

Live cell mitochondrial staining

Living cells were incubated in Mitotracker FM staining solution (0.1µM; Invitrogen) for 15 min at 37°C. Cell nuclei were stained with Hoechst 33342 (0.3µM; Invitrogen). Immediately after staining, images were taken with a Nikon Eclipse TiE microscope equipped with Perkin Elmer UltraVIEW VoX confocal imaging system (Perkin Elmer). Cropped pictures of single cells are presented.

mtDNA sequencing

B6, BALB/c, and CBA mtDNA sequencing was performed as previously described (Trifunovic et al., 2004). Results were compared to known SNPs in the Database of Single Nucleotide Polymorphisms (dbSNP; National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). dbSNP build IDs: 141 (human) and 140 (mouse) are available from http://www.ncbi.nlm.nih.gov/SNP/.

Cell transplantation

Before transplantation, NT-ESCs and isoESCs were cultured off-feeder for 1 passage to avoid contamination with feeder cells. NT-ESCs and isoESCs were trypsinated and re-suspended in sterile saline for immunobiology experiments (10^6 cells per 80µl saline for ELISPOT, 10^7 cells per 80µl saline for DTH) and NT-ESC graft survival studies (10^6 cells per 80µl saline). Cell viability was approximately 95% as determined by trypan blue staining. Cell transplantation was performed by direct injection of cell suspensions into the right thigh muscle of recipient mice using a 27-gauge syringe.

ELISPOT assays

For uni-directional Enzyme-Linked ImmunoSpot (ELISPOT) assays, recipient splenocytes were isolated from fresh spleen 5 days after NT-ESC or isoESC injection and used as responder cells. NT-ESCs or isoESCs were mitomycin-inhibited and served as stimulator cells. 10^5 stimulator cells were incubated with $5x10^5$ recipient responder splenocytes for 24h and IFNγ and IL-4 spot frequencies were automatically enumerated using an ELISPOT plate reader. Duplicates or quadruplicates were performed in most assays.

To measure the ELISPOT response to cell compartments (mitochondria or cytosol), mitochondria were isolated from $3x10^7$ NT-ESCs and served as stimulators. The remainder of the cells comprised the cytosol fraction. Successful separation of mitochondria and mitochondria-depleted cytosol was confirmed by immunoblots. Mitochondrial complex IV and GAPDH were used to confirm mitochondrial and cytosol content, respectively, and to rule out cross-contamination.

In accordance to the Medawar experiments, neonatal BALB/c mice were immunized with 10^5 mitomycin-inhibited NT-ESCs. In adulthood, the animals were re-injected with 10^6 NT-ESCs ($Balb/C_{re\text{-}inject}$) and uni-directional ELISPOT assays were performed as describe above.

Unspecifically stimulated BALB/c, BDF1, and B6 splenocytes served as ELISPOT controls. For these assays, $3x10^4$ responder splenocytes were stimulated with PMA (phorbol 12-myristate 13-acetate, Sigma, 1ng/ml) and ionomycin (Sigma, 500ng/ml) for 24 h.

Delayed-type hypersensitivity response (DTH)

DTH is a rapid T cell-dependent in vivo immune response to a foreign antigen, which the host immune system has experienced in the recent past. In the sensitization phase, $2x10^6$ NT-ESCs were injected subcutaneously into the flank of BALB/c mice. Seven days after sensitization, heated mitochondria or cytosol fractions, obtained from 10^6 NT-ESCs, were intradermally injected into the footpad during the challenge phase and paw swelling was measured after 24h using a digimatic micrometer (Mitutoyo, Aurora, IL). Results are demonstrated as swelling response to the antigen challenge in comparison to the PBS-injected contralateral control footpad of the animals.

NT-ESC-specific antibody assay using flow cytometry (FACS)

NT-ESC-specific mouse antibodies were detected by FACS analysis as previously described (Deuse et al., 2008). Briefly, the serum of BALB/c, BDF1, and B6 mice 5 days after transplantation of NT-ESC grafts was incubated with NT-ESCs and the binding of antibodies was quantified. Only IgM antibodies were analyzed because of their known rapid surge within 5

days after allogeneic stimulation. IgM antibodies were stained by incubation of the cells with a PE-conjugated goat antibody specific for the Fc portion of mouse IgM (BD Biosciences, Franklin Lakes, NJ). Cells were washed and then analyzed on a FACSCalibur system (BD Biosciences). Fluorescence data were expressed as mean fluorescence intensity (MFI).

Survival of NT-ESC grafts

Survival of NT-ESC grafts and teratoma development were assessed in vivo by injecting 10^7 NT-ESCs or isoESCs in 80µl saline intramuscularly into the indicated recipients. Cell survival leading to teratoma formation was monitored every third day for 30 days using the digimatic caliper (Mitutoyo). Usually, teratoma formation occured within 30 days. However, animals not developing teratomas were followed for 150 days to exclude late teratoma development. The overall fractions of animals that developed teratomas are shown in separate adjacent graphs.

Statistics

In box blot graphs, the median is shown, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points. In scatter blots, the central mark is the median. Intergroup differences were appropriately assessed by either unpaired Student's t test or one-way analysis of variance (ANOVA) with Bonferroni's postHoc test. * $p<0.05$, † $p<0.01$.

Supplemental References

Deuse, T., Hoyt, G., Koyanagi, T., Robbins, R.C., and Schrepfer, S. (2008). Prevention and inhibition but not reversion of chronic allograft vasculopathy by FK778. Transplantation *85*, 870-877.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics *5*, 113.

Okonechnikov, K., Golosova, O., Fursov, M., and team, U. (2012). Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics *28*, 1166-1167.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlof, S., Oldfors, A., Wibom, R.*, et al.* (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature *429*, 417-423.